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PAPER NO. 10

EFFECT OF HYPEROXIA ON THE SURVIVAL OF RED BLOOD CELLS IN THE RAT USING ^{14}CO TECHNIQUE

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INTRODUCTION

The question of whether oxygen at pressures greater than that found in air at sea level can cause the breakdown of red blood cells has been investigated by a number of workers, and it is now fairly well accepted that oxygen at partial pressures greater than 1 atmosphere can indeed cause an in vivo peroxidative hemolysis, particularly in vitamin E deficient animals (Raihi, 1955; Kann, 1964). On the other hand, attempts to demonstrate hyperoxia-induced hemolysis at oxygen pressures below 1 atmosphere without equivocation have not been forthcoming. As it turns out, one of the major physiologic changes reported in the Gemini manned space flights of 3 days or longer was a decrease in the red cell mass on the order of 10% (Fischer, 1967).

Three potential causative factors which have been considered for this phenomenon are:

1. Hypodynamia
2. Weightlessness
3. The pure hyperoxic space cabin atmosphere.

Our particular interest has been directed toward obtaining a conclusive answer to the third possibility. That is, does mild hyperoxia cause the hemolysis of red blood cells in vivo?

METHODS AND MATERIALS

The primary technique used in this study was developed by Landaw (1966, 1969). It is a cohort labeling technique, based on the fact that when red blood cells are destroyed in the body and the hemoglobin is degraded, the heme ring is opened at the alpha-methene bridge carbon atom with the oxidation of this carbon to carbon monoxide. Furthermore, the unique source of the methene bridge carbons of heme is the 2-carbon of glycine (see figure 1). Therefore, for every mole of heme degraded, 1 mole of CO is produced and exhaled. Figure 1 demonstrates also that if ^{14}C glycine labeled in the 2 position is administered to an animal, it is rapidly incorporated into hemoglobin heme of newly forming red blood cells. The glycine also labels nonhemoglobin hemes of the cytochromes and other heme constituents and labels protein as well. The nonhemoglobin hemes have a very rapid half-life. The glycine-labeled protein turnover is very slow in the rat, being on the order of 50-100 days.

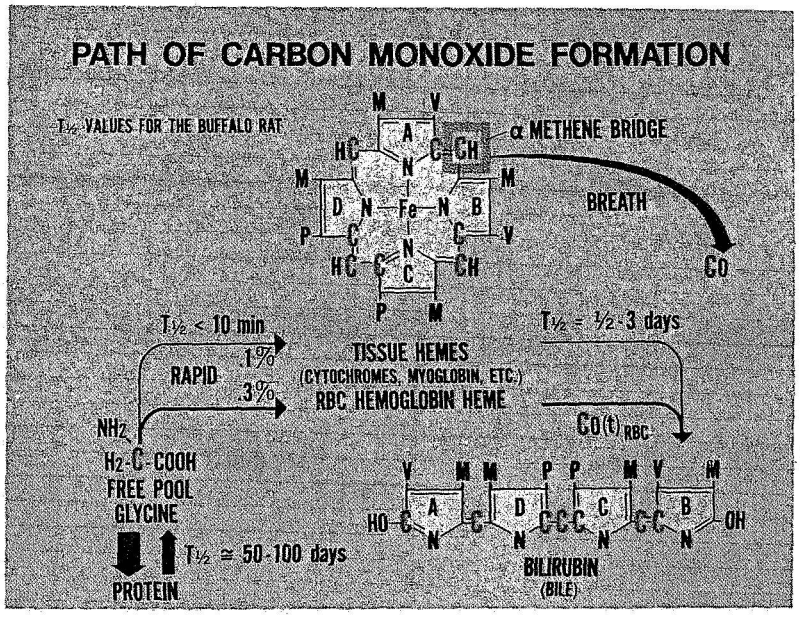

Figure 1. PATH OF CARBON MONOXIDE FORMATION.

Figure 2 shows the output of ^{14}CO from a normal Buffalo rat and illustrates how survival parameters of red blood cells can be derived. The function F (CO) $|_{total}$ describes the ^{14}CO data. F (A) and F (B) represent the ^{14}CO generated from nonhemoglobin hemes and recycling of 2-^{14}C-glycine from protein into heme. If these are subtracted from F (CO) $_{total}$ then F (CO) $_{RBC}$ which describes ^{14}CO output attributable to RBC degradation, is obtained. From this function the survival parameters of the population of RBC in question can be extracted. As one would expect the ^{14}CO output is in fact describable as a function of the circulating radioactive hemoglobin content.

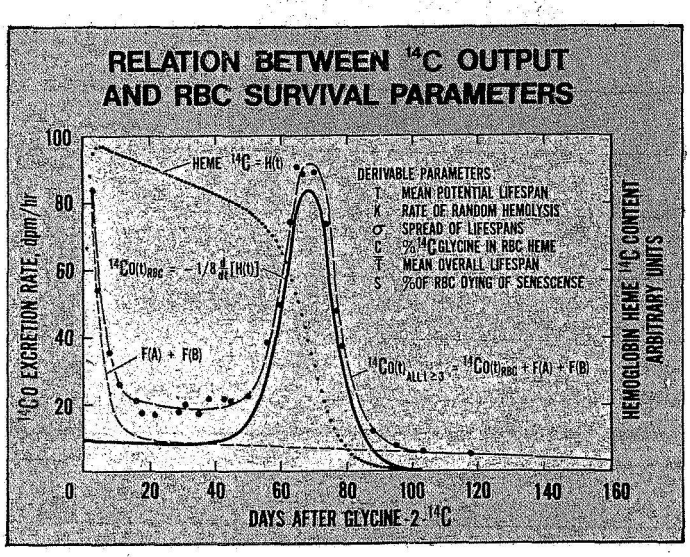

Figure 2. RELATION BETWEEN ^{14}C OUTPUT AND RBC SURVIVAL PARAMETERS.

The parameters of interest which are obtainable in this fashion are:

T - mean potential lifespan
k - the rate of random hemolysis
σ - the spread of lifespans about T
$\overline{T}$ - the mean overall lifespan
C - the percent of the injected glycine dose incorporated into RBC heme.

The capsule system for exposing rats continually to oxygen for long periods is shown in figure 3 (Quattrone, 1966). Also shown is the train for ^{14}CO collection (Landaw, 1966).

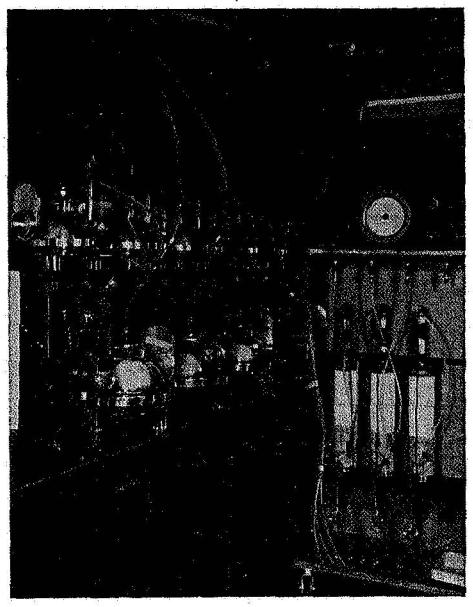

Figure 3. EXPOSURE SYSTEM AND ^{14}CO COLLECTION TRAIN.

RESULTS AND DISCUSSION

In our studies on hyperoxia we wanted to know:

1. Does oxygen affect RBC as they are being formed?
2. Does oxygen affect RBC formed under normal circumstances? If so, does the effect depend on the age of the RBC at the time of the initial exposure?
3. In either case, should there be an effect, is it dose related? That is, is there a threshold for the effect, and does it become more pronounced with increasing PO_2?

In our initial studies the following groups were studied:

1. RBC cohorts labeled in normal rats which were placed in oxygen 14 days later at 197, 258, or 450 torr. ^{14}CO output was determined under these conditions for 80 days.
2. Rats placed in oxygen at 197, 258, or 450 torr for 2 weeks and then a cohort was labeled. The rats were placed in their respective oxygen environments, and ^{14}CO was measured for 92 days.
3. Control rats labeled at the time of the other rats and ^{14}CO measured for 92 days in an air environment under sea-level conditions.

The results are listed in table I. The most remarkable finding is that the mean potential lifespan of young RBC exposed to pure oxygen for extended periods is shortened significantly. As we can see, all groups exposed to the oxygen when the labeled cells were 14 days old showed this effect. Thus, even those exposed to 197 torr, which is normoxic with respect to the alveolar partial pressure, are affected. One interpretation that can be made from this is that pure oxygen at a given partial pressure has a greater physiologic effect than in the presence of a diluent. That this may be the case is also indicated by the combined results of the Gemini and Apollo flights with respect to the red cell mass changes, for it appears that only when astronauts were exposed to pure oxygen for some time were decreases in the red cell mass noted (Fischer, 1969).

TABLE I

EFFECT OF O_2 PRESSURES ON PREFORMED AND FORMING RED BLOOD CELLS

	N		Mean Potential Life Span T (Days)	Random Hemolysis k (% day)	Spread of Life Spans σ (days)	Fraction Incorporated C (% of dose)
	(4)	Control	69.2±.9	.69±.08	6.4±.7	.265±.031
	(4)	197 torr	*66.0±.8*	.65±.07	8.0±.2	.342±.024
AIR	(4)	258 torr	*65.9±1.1*	.62±.03	7.8±.3	.260±.021
LABELED	(4)	450 torr	*63.8±.3*	.77±.05	7.6±.3	.269±.015
	(12)	Pooled	*65.2±.5*	.68±.03	7.8±.2	.290±.015
	(3)	197 torr	68.4±.4	.83±.09	8.1±.1	.215±.021
OXYGEN	(4)	258 torr	68.8±.2	.68±.03	7.1±.8	.202±.017
LABELED	(5)	450 torr	66.7±1.0	.83±.06	6.4±.3	*.161±.014*
	(12)	Pooled	68.0±.4	.76±.04	7.2±.3	*.192±.011*

Italic type indicates a significant difference from control. $P \leq 0.05$

The control mean potential lifespan was 69.2 days. In pure oxygen the value was 66.0 days in 197 torr, 65.9 days in 258 torr, and 63.8 days in 450 torr. This trend suggests a dose effect.

There was some effect on the spread of lifespans about the mean, σ, and it was significant for the pooled data. This indicates that the effect of oxygen on the mean potential lifespan is a function of the normally expected mean potential lifespan. There was no effect on the rate of random hemolysis. The fractional incorporation of glycine was normal in all instances, as is to be expected since the rats were injected with the glycine while still under normal conditions.

In view of the effect of oxygen on the mean potential lifespan of circulating red blood cells, the lack of any effect of oxygen on forming red blood cells (oxygen labeled group, table I) is rather surprising. The percent random hemolysis and the spread of lifespans appears normal in spite of continuing exposure to oxygen. One possibility is that RBC formed during hyperoxic exposure are more resistant to the hyperoxia. If this is the case, one might expect that such cells would have a greater longevity under normal conditions where the oxidative stress is lessened.

What we have seen thus far is an effect of long-term exposure to oxygen on formed red blood cells. In another set of experiments we wanted to determine the effect of acute exposure on older RBC nearing the end of their normal lifespan. In this case we used an O_2 pressure of 600 torr. The results of this experiment are illustrated in figure 4. The rats were labeled on Day 0 and placed in capsules in air on Day 40. On Day 55, after the senescence process had started, the rats were switched over to oxygen. It can be seen that there appears to be an almost immediate increase in the output of ^{14}CO indicating an increased rate of hemolysis of the older cells. This spike represents about 11% of the cells potentially at risk on Day 55. Thus it appears that O_2 can indeed cause the lysis of red blood cells and this effect appears to have some relationship to the age of the cells at the time of the initial exposure.

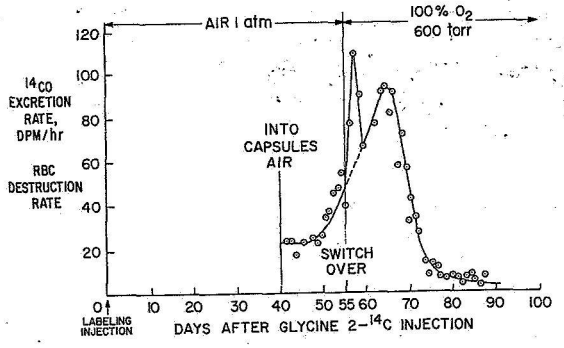

Figure 4. EFFECT OF OXYGEN AT 600 TORR ON DESTRUCTION RATE OF SENESCENT RED BLOOD CELLS.

The normalization of the senescence process after 3-5 days and the lack of any effect of continuing oxygen exposure on the survival parameters of labeled cohorts formed after 2 weeks of oxygen exposure could be interpretated to mean that the oxygen was not directly affecting RBC. In order to obtain a direct answer to this question the experiments listed in table II were performed. Normal rats were kept in capsules in air at sea level or in oxygen at 600 torr for 5 days since earlier experiments indicated that this period of time is required for the maximal effects of oxygen to be expressed. The rats were then infused with 2.0 ml of washed RBC from rats labeled with 2-^{14}C-glycine 53 days previously. Thus these cells were already beginning to undergo senescence. The rats were then split into 4 groups and immediately placed in the respective environments indicated. The formation of ^{14}CO was then measured. From these results it is clear that regardless of the previous condition of the rat, exposure to oxygen increases the breakdown of red blood cells and is measurable within the first 5 hours of exposure. Conversely, rats with an immediate prior history of exposure to oxygen do not respond differently from normal animals either to oxygen or to air. It is concluded from these findings that the effect of oxygen on the RBC is a direct one, and no residual effect indicative of tissue changes is demonstrable.

TABLE II

EFFECT OF PRECEDING AND IMMEDIATE ENVIRONMENTAL CONDITIONS ON ^{14}CO OUTPUT FROM INFUSED RBC LABELED 53 DAYS PREVIOUSLY

Initial Exposure Condition (N)	Immediate Postinfusion Exposure Conditions	^{14}CO Output dmp/hr 1st 5 Hrs Postinfusion	Significantly Different From
1. Air, 760 torr (6)	Air, 760 torr	24.6±1.5*	2, 4
2. Air, 760 torr (4)	O_2, 600 torr	39.3±3.0	1, 3
3. O_2, 600 torr (6)	Air, 760 torr	18.9±2.2	2, 4
4. O_2, 600 torr (6)	O_2, 600 torr	34.8±2.7	1, 3

*S.E.M.
Mature male Buffalo rats were exposed to air at sea-level or to pure oxygen at 600 torr for 5 days. Then, 2.0 ml of pooled and washed RBC from donor rats which had been injected with 2-^{14}C-glycine 53 days, previously were infused into each rat. The rats were immediately placed in the indicated postinfusion environment and 5-hr samples of ^{14}CO were collected.

In conclusion, mild hyperoxia causes hemolysis of rat red blood cells in vivo, and shortens the lifespan of cells exposed for prolonged periods.

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DISCUSSION

MAJOR THEODORE: I just have a few questions. First, have you done iron kinetic studies or iron tagging to see if these match up with your data as well as for as survivability, seeing where the cells are going, whether they are having intravascular hemolysis, whether the cells are damaged and being picked up by the spleen, or are they actually being destroyed in the marrow?

DR. LEON: No, we haven't done iron kinetic studies. Dr. Winchell, who is a foremost expert on iron kinetics has done the iron kinetics on the aquanauts in the Sea Lab III, and in this case, there was an inert gas present even though the environment was hyperoxic, and he found no changes in the iron kinetics. We haven't done the things you suggest. We have done other types of experiments with osmotic fragility and red blood cell enzyme changes where the data is in line with what we found here. The osmotic fragility studies indicate that you have a younger population which would follow if you knock off the older ones, and the enzyme studies done by Dr. Sabin in our lab indicate the same thing. Within three days you get an increase in G6PD and hexokinase, and the only way you can get an increase is to have a younger population.

MAJOR THEODORE: Is this due to increased marrow activity or does the marrow not respond then?

DR. LEON: No, apparently--

MAJOR THEODORE: Iron uptakes would be accelerated under points of intravascular hemolysis.

DR. LEON: Well, all evidence seems to indicate that there is a suppression of erythropoiesis that was indicated by our decreased uptake of the glycine and other people in the past have done work which supports the idea that hyperoxic exposures suppress erythropoiesis. In view of that, if you accept that as being true, the only way to get an increase in an enzyme in a non-nucleated cell like the red blood cell, or the only way that you can get an increased resistance to osmotic fragility would be to have a younger population and the only way that you can get a younger population under these conditions, at least the only way that I can imagine, is to knock off the older ones.

MAJOR THEODORE: That's true. This is a beautiful technique using the glycine tag in the two position evolving carbon monoxide, but I was wondering here in the methodology, how do you correct a CO washout effect? By this I mean over this period of 60 days you're measuring peak CO output. During that time you may have some

CO leak that's now bound to hemoglobin--not incorporated, but bound--and I think some of the peaks you got initially upon exposure to oxygen may well be just the competitive release of carbon monoxide from the heme which is in actual competition with oxygen for the same site. I think this is a moot point. Out of curiosity I was wondering if you were able to correct for this CO output versus that which is being evolved when you break down your heme.

DR. LEON: Well, that bothered me at first when I first was introduced to this technique, because you always hear that carbon monoxide binds to hemoglobin two to three hundred times greater than oxygen. This is true, but, in fact, it's in dynamic equilibrium. For example, we have done some early studies, early peak studies, where you inject the glycine and immediately after injecting the glycine you start to take your carbon monoxide samples, and from this you can determine turnover rates of your cytochromes and you can determine the quantity of ineffective erythropoiesis and you can determine the extent at which little driplets of hemoglobin come out of the retics on the mitochondrion, and so forth. What you see is that you inject the glycine, and within seconds you're getting your carbon monoxide out and, in fact, the peak of the carbon monoxide output from the cytochromes is at a half hour after the injection. So, you're right that it's bound tightly to the hemoglobin, but it's just like it comes in and pushes it out. It goes real fast. It becomes mixed in rapidly, within seconds, and it's out in proportion to its quantity, very rapidly.

MAJOR THEODORE: I just have one more question and then I'll quit. Has anyone looked at the marrow, per se, by electron microscopy with those techniques where one wants to look at the red cell mitochondria, since hemoglobin synthesis in the red cell precursors occurs at these sites? When you're talking about things like sideroblastic anemias, etc. where they demonstrate definite mitochondrial lesions, I was wondering if this type of model or analogy was looked at from this basis, since you have presented evidence that there may be some abnormality of incorporation. If one wants to stretch his imagination a bit one could look at this as a sideroblastic type of problem and therefore maybe looking at the mitochondria and seeing if there are changes, but this is in the marrow cells because as the cells maturate they lose this. This is just another point. I'm not refuting what you're saying; I'm saying some things that maybe one could look at--

DR. LEON: I don't think--I wouldn't call it abnormal; I would just call it decreased.

MAJOR THEODORE: That's abnormal. You're losing 20% of your red cell mass, if I understand the data on the astronauts. I think that's significant.

DR. LEON: Well, it's significant, but it may just be physiological. I like to differentiate between a physiological mechanism and an abnormal mechanism. You can accelerate and decelerate a physiological mechanism and still not be abnormal.

MAJOR THEODORE: That's because our people have recovered.

DR. LEON: For example, if you go to high altitude you've got an accelerated production of red blood cells, and this certainly isn't abnormal; it's a normal reaction. That's the point I was making, but I don't know that anybody has looked at the red blood cells. I think there are people who are very interested in doing it and maybe some of the electron microscopists in the group might be able to comment on that.

CAPTAIN CHIKOS: The only question I had was you are demonstrating a decrease in the red cell population, and when one uses a term hemolysis you usually imply a pathological state of intravascular lysis of red blood cells, and I think it would be more appropriate to say that you're just having a decreased survival time for whatever cause. It might be just accelerated destruction by the spleen in this case, or some other physiological mechanism.

DR. LEON: Yes, I don't disagree with you. Actually, this technique is extremely sensitive and it has been difficult for us to actually demonstrate, in these particular cases, a decreased red cell mass, although we are going to try to do this next.

DR. CAMPBELL: I was wondering if anyone could comment on the possible formation and effects by peroxides in these situations, and also, I might disagree with the allusion made that the only mechanism for red cell fragility might be younger cells, because I should think that possible direct effects on red cell membranes could also result in this without there being an effect on the age of the cell.

DR. LEON: Well, with respect to that latter point, I would say that I don't know of any instance where some toxic or peroxidative agent increases the resistance of red blood cells to osmotic fragility. It usually decreases their resistance, so--you're right. This is a weak point, and actually, we are working on the osmotic fragility quite heavily, trying to show that the osmotic fragility changes when the osmotic fragility decreases, that it indicates a younger population. We haven't really proven that to our satisfaction as yet.

MAJOR CASEY (School of Aerospace Medicine): I didn't get how you ruled out a stimulatory effect on the RE system or spleen, that it was a direct effect on the red cells themselves.

DR. LEON: Well, the rats that were exposed to oxygen at 600 torr for 5 days prior to the infusion of the 53-day old cells, when they were placed into air, they actually had a lower breakdown of red blood cells, as indicated by the carbon monoxide output, than the normals. The red blood cells that were infused, the ones that were 53 days old, came from normal rats, that is, these rats had just been kept in air, so these were untreated red blood cells other than the fact that they were radioactive. These rats had been in oxygen for five days and then they were immediately injected with 2 mls of the 53-day old red blood cells and they were put in air right after and the carbon monoxide output was determined. The value was actually lower than in the normal control, but statistically, they were the same, so that you can say that the breakdown of red blood cells in this group which had been conditioned to the oxygen was no different than normal controls. So there is no indication from this that the RE system had been stimulated. If it had been stimulated, it was an on-off stimulation.

MAJOR CASEY: In this circumstance, don't you get blood mismatches in rats as well? How much hemolysis was induced here by antibodies? You're taking blood from one animal and putting it in another.

DR. LEON: These are pretty homogenous rats.

MAJOR CASEY: I don't know how homogenous rats are; that's what I'm asking.

DR. LEON: Nevertheless, this experiment is a controlled experiment, and you'd see it. This is your control, and part of this output here is actually due to a breakdown of some damaged red blood cells, taking them up in the syringe and infusing them, so this would form a control on that question that you raise.

This is a normal control, 24.6. This was the air that was placed in the oxygen. It's 39.3 and it is significantly higher. These are the ones that had been in oxygen and were put into air and they're 18.9. They're not significantly different from the controls, but they are significantly lower than this group here, and this is 34.8. These were in oxygen initially and were put in oxygen subsequently, so no matter what the prior condition was, if they were put into oxygen they had high outputs of carbon monoxide, and no matter what the prior condition was, if they were put into air they had low carbon monoxide outputs.

DR. WEIBEL: Well, I don't want to take too much time. I'm going to put some of the comments I wanted to make in my paper this afternoon where you shall see that we have found essentially data on the changes in the blood at one atmosphere of pure oxygen in rats which are quite in keeping with the data that Dr. Leon showed. Actually, I'm very happy about his findings because as you know they are somewhat different from what other people have said. We have felt a little uneasy about that. I was a little worried about your comment that the red cell erythropoiesis would be depressed. Is this really supported by evidence? Because we found that there was no decrease in red cell mass; on the contrary, you will see that hemoglobin and hematocrit increased after 48 and 60 hours in pure oxygen. Of course, you may have the question that this is probably due to fluid loss, but still for two days when you get no change and where you get no edema formation, you have very stable hemoglobin and hematocrit in these rats. Well, I have just one question. What was the time in your second experiment until this extra peak occurred? This was a little compressed on the slide, you know. The one you gave after 55 days of pure oxygen. What was the time of the peak?

DR. LEON: We put them into oxygen and immediately started the determination and we had to take a five-hour determination because there's not much carbon monoxide coming off. In fact, in a man, a man gives off 0.4 cc per hour so you can imagine how much a rat gives off, and we are just measuring the radioactive carbon monoxide. During the first five hours the first point was essentially on the normal curve. The second day it was already above the baseline, so it's at least within 24 hours. Now, our last experiment indicates that it happens immediately within the first five hours.

DR. WEIBEL: Yes. I'm very happy about that.

COLONEL MUSGRAVE (USAF School of Aerospace Medicine): You seem to have a unique method here and a pretty good fix on the red cell life in these rats and I just wonder if you have, or plan, to look at the reverse situation under hypoxia to determin whether the characteristic response is entirely hematopoietic or whether you actually prolong the life span of the red cell using this technique.

DR. LEON: As a matter of fact, Dr. Landau is doing considerable work on this. He is in the Donner Laboratories at Berkeley, he and Dr. Winchell are doing considerable work on this. They are also doing considerable work using this technique on stressed red blood cells, that is, cells produced in reaction to hemorrhage, cells that are produced real quickly in response to some stress, and they show, in this case, that the mean potential life span is progressively shortened, depending on the severity of the stress or the amount of hemorrhage that is induced. They are doing work on hypoxia but in that case they do not find an increase in the mean potential life span. However, in splenectomized rats, they find an increase of four days in the mean potential life span, so the spleen is a selective organ and it has a selectivity of about four days. They do find an increased mean potential life span in hyp'd animals, but in this case you don't know if it is because of their decreased metabolism or what, and they are working on that right now.

DR. BENJAMIN: Dr. Leon, your findings have some significant effect on space flight insofar as at present astronauts are exposed to some 800 mm of mercury pure oxygen during the launch for several hours. This would indicate that there would be significant changes of red blood cells which we really did not account for so far. Do you have any plans to determine the effect in men?

DR. LEON: Well, in the first place, they also had nitrogen in the atmosphere.

DR. BENJAMIN: No, they do not, not during the launch. You see the man is in the suit, in a pure oxygen atmosphere at about 800 mm of mercury. The cabin of the spacecraft itself is a mixed gas atmosphere, 60% nitrogen, 40% oxygen.

DR. LEON: How long are they in this environment?

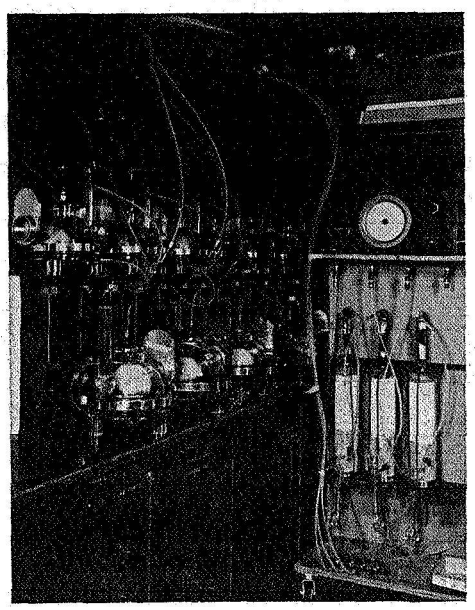
DR. BENJAMIN: Something like three or four hours.

DR. LEON: Well, Craig Fisher has invited us down next month to talk about doing some work, and I don't know what the plans will be, certainly, it's worth keeping what you said in mind. I assumed that they had been in a mixed gas environment.

DR. COULSTON: Let's get one thing straight, will you? You're working with a rat. A rat produces extramedullar hematopoiesis all over the place. I would suggest very strongly that some histopathology be done and that you pick a species that is more like man and not extrapolate too much from the rats to men. I think the experiment is beautiful. It's a nice experiment. But put a monkey in a little capsule. He doesn't have extramedullar erythropoiesis all over the place. This may explain your young

population of cells very easily, because the mobilization of these young cells can be very quick--under the terms of all the torrs that you used and the oxygen you used. I don't know if this is correct but it could be.

DR. LEON: I agree with you, we should do it on another species. We hope we can do it on man.



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